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The water adsorption characteristics of charged phospholipids

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Abstract

The hydration isotherms of the negatively charged phospholipids, egg phosphatidic acid, bovine heart cardiolipin and two phosphatidylserines as well as one positively charged phospholipid, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine, have been obtained gravimetrically. The presence of an electrical charge on these phospholipids does not, in itself, determine whether the water binding to the phospholipids is 'strong' or 'weak'. Interestingly, hysteresis effects were present for certain charged phospholipids suggesting some rearrangement of the lipid molecular organization upon hydration and dehydration, perhaps due to the presence of the ionizable moiety. The hydration isotherms of the charged phospholipids have been analyzed by BET theory, although, of the charged lipids studied, all may not be amenable to the application of BET theory. The hydration isotherms and the resulting parameters obtained from the BET analysis are compared to those found previously for zwitterionic phospholipids, especially egg phosphatidylcholine. The water adsorption characteristics of phospholipids are found to depend mainly on the total head-group structure including the presence of hydrophobic groups as well as electrical charge on the head-group.

Keywords: Phospholipid hydration; BET theory; Charged phospholipid; Physical adsorption

1. Introduction

The nature of the short-range forces between amphiphilic surfaces, such as those in phospholipid aggregates, is an active research field. This strong interest is directly related to the application of the concepts involved to biological systems such as cellular membranes. Theories and experimental results concerning these forces have been reviewed in recent articles [1–3].

There are various ideas as to the origin of these forces in biological systems which, because of the presence of water, involve the interaction of the water with the amphiphilic surface. One view is expressed in the 'solvent polarization model' which involves the interaction of the surface with water in a local orientation of the water molecules near the lipid-water interface [4]. It is noteworthy that Ellworthy was one of the first to study lipid hydration [5,6]. In the work described in this publication we present the results of hydration studies of electrically charged phospholipids. In particular, we have obtained the water adsorption isotherms for EPA, BPS, BCL and DOEP. We have applied BET theory [7] in our analyses of the isotherms for these lipids and contrasted the BET results with those obtained from our earlier published isotherms for egg phosphatidycholine [8].

BET theory, when applicable, allows the calculation of the binding energies of the vapor to the adsorbing surface as well as the number of molecules in the first 'monolayer' of adsorbate on the surface [7]. We also present our ideas as to how the presence of the net electrical charge on the phospholipids affects the water binding vis-à-vis the water binding for zwitterionic phospholipids. A second paper (unpublished data) describes the use of electrical conductivity measurements of these hydrated, charged phospholipid systems so as to provide further information on the nature of this hydration process. Although we do not attempt to resolve the problems of choosing one or the other theory of hydration forces as valid, nevertheless, we believe our hydration results are intimately related to the development of such theories. Additionally we believe that the first few bound water molecules lead to the appearance

Abbreviations: BCL, bovine cardiolipin; BPS, bovine phosphatidylserine; DOEP, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine; DOPC, dioleoyl phosphatidylcholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-(phospho-L-serine); EPA, egg phosphatidic acid; EPE, egg phosphatidylethanolamine; PA, phosphatidic acid; PMME, phosphatidylmonomethylethanolamine.

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of these hydration forces and their ultimate biological effects.

2. Materials and methods

The lipids examined in this work were obtained from Avanti Polar Lipids, Inc., Alabaster, AL. The lipids were of greater than 99% purity and no additional purification was used. Until the samples were deposited on the Teflon substrates, storage was under prepurified nitrogen at −70°C.

The hydrocarbon chain composition of the natural phospholipids is as given by Avanti Polar Lipids [9]. In particular, the fatty acid content for EPC, EPA and EPE is about 34%, 16:0; 11%, 18:0; 31%, 18:1 and 18%, 18:2. BPS contains 40%, 18:0 and 31%, 18:1 fatty acid composition whereas cardiolipin contains more than 90%, 18:2 unsaturation.

The experimental technique of using a microbalance as a gravimetric means of measuring physical water adsorption characteristics has been previously reported [10]. A Cahn G-2 electrobalance in the 'remote weighing' mode provided a resolution on the order of 10^{-6} g. The experimental apparatus is a somewhat modified version of that utilized in this earlier work. The temperature during the adsorption process was held to 22.0°C ± 0.2°C as indicated by an Omega 670 digital thermocouple thermometer with a type T probe.

After a sample was deposited on a substrate it was normally placed within an aluminum chamber and dried under vacuum. It was then moved to the weighing chamber, placed on the balance stirrup and dried under prepurified nitrogen flow until its weight ceased to change. In some cases the samples were placed directly in the weighing chamber under nitrogen flow, again until the weight ceased to change. This constant weight value is defined as the sample's 'dry weight'. Most sample dry weights were 2 to 4 mg.

Controlled hydration was accomplished by enclosing vessels of saturated salt solutions in the chamber and then purging the system with prepurified nitrogen. The chamber was allowed to reach equilibrium (approx. 24 h) for each hydration determination. More experimental details are available in our previous work [10].

The apparatus included a second sample of the phospholipid deposited on a one by one-sixteenth inch quartz disk. This sample was positioned between and in contact with two 5 mm × 5 mm vacuum deposited electrodes separated by 5 mm. Application of a 2 V direct current source to the sample from the internal power supply of a Keithley 617 programmable electrometer provided concurrent electrical current measurements which were later converted to conductivity values. This electrical technique proved to be a most sensitive indicator of the hydration state of the sample.

3. BET theory (multimolecular adsorption theory)

BET theory is based on the five isotherm types found in the literature. These 'idealized' isotherms are shown in Fig. 1. The multimolecular adsorption theory, in an attempt to give a unified theory of physical adsorption and its most general equation, includes all five isotherm types shown in Fig. 1 as special cases and moreover describes the shape of each isotherm type over the entire range of vapor adsorption, from zero to the saturation vapor pressure.

The fundamental assumption of BET theory is that the same forces that are active in condensation are also those producing the adsorption of the gas on the adsorbent surface. Using this assumption and some lengthy algebra, the adsorption isotherm equation is obtained:

$$\frac{p}{v(p_0-p)} = \frac{1}{v_m c} + \frac{c-1}{v_m c}\cdot\frac{p}{p_0} \qquad (1)$$

This is a linear equation and a plot of $p/v(p_0-p)$ vs. p/p_0 gives a straight line, if the theory is obeyed. Using the intercept of the straight line, $\frac{1}{v_m c}$, and the slope, $\frac{(c-1)}{v_m c}$, the constants v_m and c can thus be obtained from the experimental data. Here v is the volume of gas adsorbed at the vapor pressure p; v_m is the volume of gas adsorbed in a complete unimolecular layer of the gas molecules. p_0 is the pressure of the gas at saturation and $c = \exp(E_1 - E_L)/RT$ where E_1 is the average heat of adsorption of the gas in the first adsorbed layer and E_L is the heat of liquefaction. Further details of this approach can be found in the elegant work of BET theory [7]. Isotherm types III and V are thought to indicate weak water binding, i.e., $E_1 \leq E_L$ whereas types II and IV indicate strong water binding, i.e., $E_1 > E_L$. Note that what differentiates a strong from a weak water binder is the shape of the isotherm below p/p_o values of about 0.5.

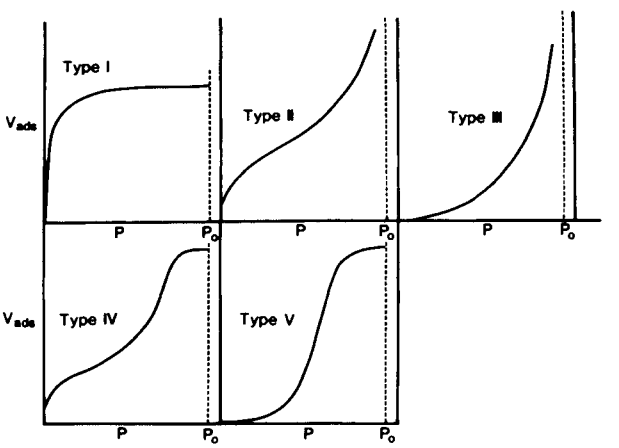

Fig. 1. Idealized adsorption isotherms. These isotherm types are illustrative of those found in the literature for a variety of adsorbates and adsorbents.

4. Results

In Fig. 2 are shown the adsorption isotherms for BCL, EPC, DOEP and BPS. Clearly the BPS isotherm is a type V isotherm according to Fig. 1 and indicates weak water binding by BPS. In contrast the isotherm for EPC is a type IV isotherm indicative of strong water binding. EPC is electrically uncharged whereas BPS carries a negative charge. The difference in the shapes of the two isotherms is evident.

The positively charged DOEP isotherm is a type IV, however, the amount of water bound by DOEP, although greater than that bound by BPS, is significantly less than that bound by EPC. Furthermore, comparing the DOPC isotherm (not shown) with that for DOEP, reveals an even greater adsorption difference since DOPC binds somewhat more water than does EPC nevertheless the DOPC isotherm is quite similar in shape to that for EPC.

The isotherm for BCL is also type IV but shows significantly greater water adsorption by BCL than by EPC. A BCL molecule contains two PA molecules and its isotherm should be compared with that for EPA shown in Fig. 4.

In Fig. 3 is shown the BET plot for BCL. This plot is obtained by applying Eq. 1 to the BCL isotherm shown in Fig. 2. The BET plot shown is a straight line from about p/p_0 near zero to p/p_0 approximately 0.35. Such a good straight line plot over this p/p_0 range provides strong support for the applicability of BET theory to BCL.

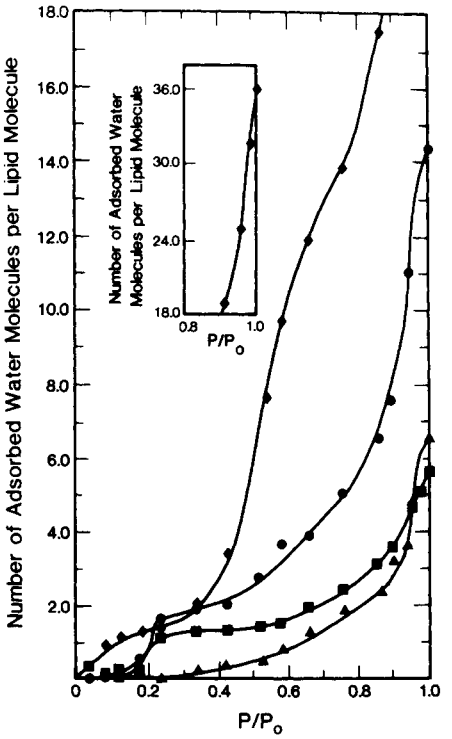

Fig. 2. Water adsorption isotherms at 22°C. ◆-◆, bovine heart cardiolipin; ●-●, egg phosphatidylcholine; ■-■ 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine; ▲-▲ bovine phosphatidylserine.

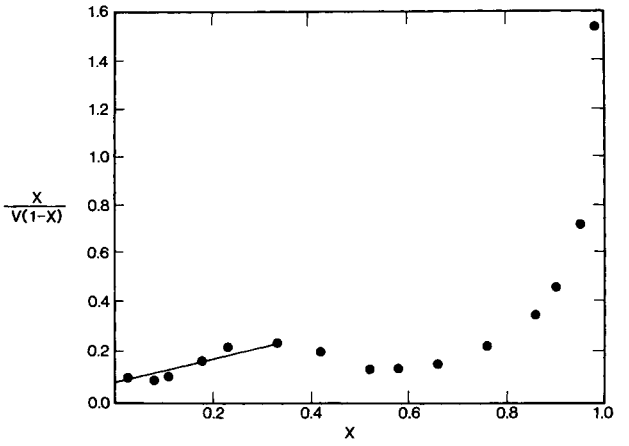

Fig. 3. Adsorption isotherm data for bovine cardiolipin plotted according to the BET Eq. 1.

In Fig. 4 are shown both adsorption and desorption isotherms for EPA. Note that the adsorption isotherm is type IV whereas the desorption isotherm is essentially a straight line below partial vapor pressures of about 0.8. This isotherm difference indicates a hysteresis process as the water is desorbed. It has not been possible in our hands to remove all the water adsorbed during the adsorption process from p/p_0 of zero to approx. 100%. This is evident from the greater bound water values of the desorption isotherm as compared to the adsorption isotherm. Our electrical measurements are also consistent with this hysteresis effect; these electrical measurements will be reported in a later publication.

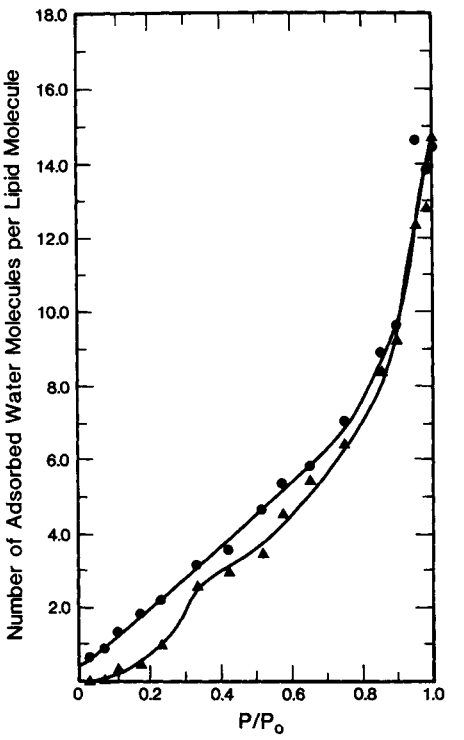

Fig. 4. Water adsorption isotherm (▲-▲) and desorption isotherm (●-●) for egg phosphatidic acid at 22°C.

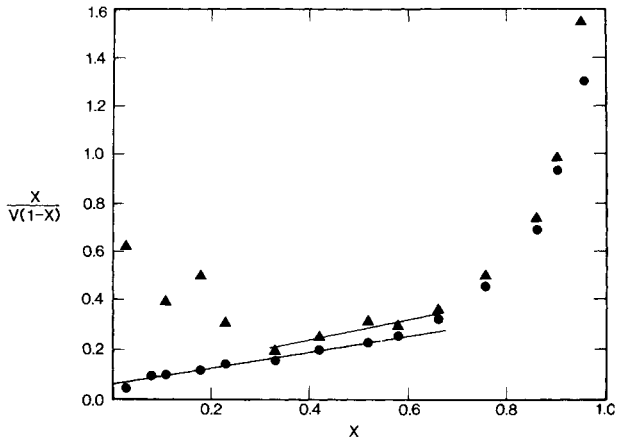

Fig. 5. Adsorption (▲-▲) and desorption (●-●) isotherm data for egg phosphatidic acid plotted according to the BET Eq. 1.

Fig. 5 displays the BET plots obtained from both the EPA adsorption and desorption isotherms shown in Fig. 4. These plots were obtained by applying Eq. 1 to the respective adsorption and desorption isotherms. A significant straight line BET plot is obtainable from the adsorption isotherm only for p/p_0 values from about 0.33 to 0.7. For the desorption isotherm, however, a straight line BET relationship is obtained for p/p_0 values from 0.7 to zero. The fact that a straight line BET plot may not be attainable below a p/p_0 value of about 0.33 makes the application of BET theory to the EPA adsorption process somewhat questionable since BET theory is usually applied to adsorption at lower p/p_0 values [7] than 0.33, however, we have applied BET theory to the EPA, adsorption process.

In Fig. 6 are displayed adsorption isotherms for EPC, EPA and a 7:4 molar complex of EPC and EPA. Note that at p/p_0 values greater than about 0.2, the EPC/EPA complex shows a greater water adsorption than does either EPC or EPA alone. All three of the isotherms are classified as type IV by the BET criteria illustrated in Fig. 1 and the individual lipids as well as their complex are thus all strong water binders.

In Table 1 are tabulated the values of v_m and E_1 calculated by applying Eq. 1 to the isotherms for the charged phospholipids studied in this work; the values obtained for EPC are shown for comparison purposes. The

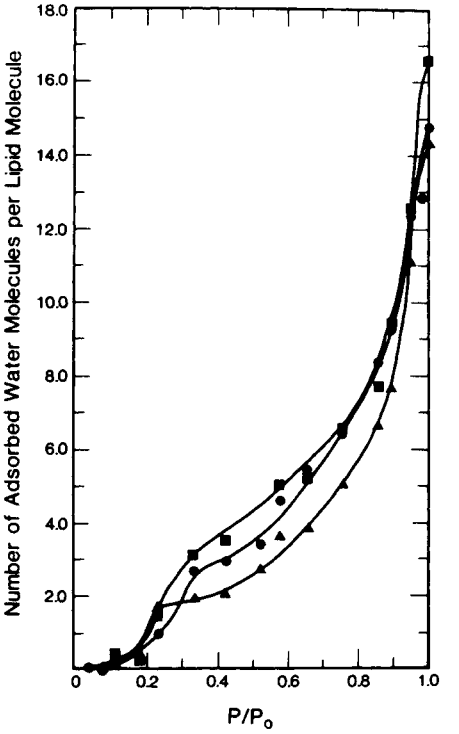

Fig. 6. Water adsorption isotherms at 22°C. ■-■, egg phosphatidylcholine-egg phosphatidic acid complex, 7:4, M/M; ●-●, egg phosphatidic acid; ▲-▲, egg phosphatidylcholine.

'r' values shown are those for the straight line sections of the BET plots for the respective phospholipids. The p/p_0 range over which this straight line relationship holds is also shown and it can be seen that the linear relationship is quite good over these vapor pressure ranges. Since BPS is a weak water adsorber the v_m and E_1 values can only be estimated for this lipid [7]. Furthermore, because of the negative intercept for the DOEP BET plot, we do not apply BET theory to the isotherm for this lipid.

The BET values and their significance are determined as follows. The water adsorption values at each p/p_0 are determined for each phospholipid for n samples. The n values at each p/p_0 are averaged and their standard deviation determined. An isotherm for these average values is then plotted and from this isotherm, an 'average'

Table 1
BET constants

Phospholipids		p/p_0 (range)	V_m (molecules water per lipid molecule)	E_1 (kcal/mol)	r
EPC	$n=7$	0.23–0.66	1.41	11.4	0.97
EPA (adsorption)	$n=6$	0.33–0.66	1.90	11.2	0.96
EPA (desorption)	$n=3$	0.03–0.58	2.49	10.8	0.99
EPC/EPA (7:4, M/M)	$n=6$	0.33–0.76	2.09	12.6	0.98
EPC/EPA (7:4, M/M)	$n=6$	0.23–0.66	2.66	10.6	0.88
EPC/EPA (7:4, M/M)	$n=6$	0.23–0.76	2.04	11.1	0.91
BCL	$n=2$	0.03–0.33	1.56	11.0	0.95
BPS	$n=3$	0.50	0.50 (estimate)	$E_1 < E_L$	N/A
DOEP	$n=3$	0.23–0.66	see text	see text	0.99

BET plot is obtained for each phospholipid. From the BET plots, v_m and E_1 values for each phospholipid are determined and those values are shown in Table 1. Other calculation procedures have been tried, however, little change in the v_m and E_1 values occur. It should be noted here that the important points are that v_m ranges from 1.5–2.5 water molecules per phospholipid molecule and E_1 ranges from approx. 10–13 kcal/mol. Our experimental techniques will have to be further refined in order to make these numbers more precise.

We have not shown the standard deviations for the isotherms of Figs. 2 and 6 since this would complicate the figures excessively. Suffice it to say, however, the conclusions of the paper are consistent with the statistical analysis. As might be expected the data at the lowest p/p_0 values show the largest percentage standard deviations because of the instrument drift and sensitivity characteristics.

5. Discussion

From the data presented here for the charged phospholipids, it is clear that the presence of the charged moiety on the phospholipid does not, in itself, predict the nature of the water binding. This is evident by comparing the adsorption isotherms shown in Fig. 2 where both type V (BPS, weak water binding) and IV (DOEP, BCL, and EPC, strong water binding), isotherms for these charged phospholipids are displayed, and in Fig. 4 where the EPA adsorption and desorption isotherms are displayed. The BPS and EPA molecules each carry a single negative charge whereas the BCL molecule carries a double negative charge; the DOEP molecule carries a single positive charge. Although the hydrocarbon chain compositions of BCL and BPS differ from that for EPA, our studies have shown that such composition differences do not determine the isotherm classification, i.e., whether the water binding is weak or strong; the isotherm classification is primarily determined by the head-group structure. The chain composition, however, does affect the amount of water adsorbed at each p/p_0 value. For example, if one compares the BPS isotherm and the DOPS isotherm (not shown), DOPS is still a weak water binder, however, DOPS adsorbs somewhat more water than does BPS. Moreover, the adsorption isotherm for lyso-BPS is found to be a type III isotherm, again indicating weak water binding [8], however, the amount of water adsorbed by lyso-BPS is much greater than that adsorbed by BPS.

From Figs. 2 and 4, it can be seen that EPC and EPA both exhibit type IV adsorption isotherms, indicating 'strong' water binding; the two isotherms are quite similar, both qualitatively and quantitatively even though the EPC molecule contains a choline group and is zwitterionic whereas the EPA molecule has no choline group and, moreover, is negatively charged. The two phospholipids do have identical hydrocarbon chain compositions. It thus can be seen that strong water binding can occur for two phospholipids even though the head-group of one is negatively charged whereas the other is electrically neutral.

BCL also displays a type IV isotherm. If this isotherm is compared with that for EPA, the BCL molecule is found to adsorb more than twice the number of water molecules, over the same p/p_0 range, as does EPA. Since BCL consists of two PA molecules 'tied together' by a glycerol element, this doubling of the water adsorbed for BCL, vis-à-vis EPA, is what might be predicted. The deviation from the factor of '2' for the water adsorption may well result from the difference in hydrocarbon chain composition between EPA and BCL which would be consistent with our earlier work [8] showing greater water adsorption for phospholipids having the same head-group but greater unsaturation of their hydrocarbon chains. As mentioned the BCL hydrocarbon chain composition is greater than 90%, 18:2 whereas EPA is about 50%, 16:0 and 18:0 and 50%, 18:1 and 18:2. This composition difference, nevertheless, does not result in different isotherm classifications; indeed, we have found that whether a phospholipid is hydrated above or below its hydrocarbon chain transition temperature does not change the isotherm classification [11]. The fact that the two PA molecules are connected via a glycerol molecule and thus their head-groups are not in the same state as in free EPA, could also alter the amount of water adsorbed at each p/p_0 in comparison with that expected for two 'non-linked' EPA molecules. Presently, the effect of cardiolipin, head-group, structure variations on water binding is being examined with cardiolipin analogs.

In contrast to EPA, BPS and DOPS display weak water binding adsorption isotherms (type V); EPE, a zwitterionic phospholipid, is the only other phospholipid found by us so far, to display a type V isotherm. EPE and BPS both have mixed hydrocarbon chain compositions, and their respective isotherms are similar both quantitatively and qualitatively. This isotherm similarity again argues for our suggestion that the presence of a net electrical charge on the head-group is not the primary determinant of the nature of the water adsorption.

To illustrate the complexity of water adsorption, EPE displays a type V (weak water binding) isotherm; PMME, however, which has but one methyl group attached to its N^+ moiety, displays a type IV (strong water binding) isotherm [11]. EPC also exhibits a type IV isotherm and the result of the two additional methyl groups attached to its N^+ element is increased water adsorption compared to PMME; the isotherm classification, is, however, unchanged from that for PMME [11]. EPA on the other hand, has no choline element in its head-group yet the adsorption isotherm for EPA is similar to that for EPC, both qualitatively and quantitatively. This isotherm similarity is also true for the EPC-EPA complex shown in Fig. 6. It thus appears that the water adsorption process for a phospho-

lipid depends on a number of factors such as the presence of hydrophobic groups and the net electrical charge in the head-group structure.

The weak, water-binding characteristics of EPE, may well be due to intra/inter molecular hydrogen bonding whereas the presence of even a single methyl group, as in PMME, may interfere with such bonding, thereby resulting in strong water adsorption [12]. The negative charge in EPA may also hinder such hydrogen bonding, the consequence again being strong water adsorption, even though no methyl groups are present in the EPA head-group. In an excellent review Boggs [12] develops a scheme comparing ionization state, hydrogen bonding and hydration for various phospholipids; the hydration values for EPA and certain other charged phospholipids had not all been determined at that time, however, and further analysis will be necessary to see if this scheme is consistent with the present hydration data although preliminary analysis indicates that our results are consistent with this scheme.

The isotherm for the positively charged DOEP, shown in Fig. 2, is rather different. This isotherm is indicative of strong water binding, however, the amount of water bound to DOEP, over the same p/p_0 range, is significantly less than that adsorbed by the other strong-water-binding phospholipids, even though DOEP does contain a choline group. If DOPC, another strong water binder [8], rather than EPC is used for comparison purposes, thus keeping the hydrocarbon chains identical, the difference between the amount of water adsorbed by the DOEP and DOPC is even greater than that between DOEP and EPC since DOPC adsorbs somewhat more water than does EPC. If a BET plot is constructed from the DOEP isotherm, a negative value for the intercept is obtained and BET theory may not be applicable to DOEP. This negative intercept is unique for the phospholipids we have studied and it remains to be seen whether it is related to the presence of a positive charge. The anomalous behavior of DOEP is also shown in our electrical conductivity studies: the relatively high conductivity for the 'dry' DOEP relative to that of the other charged dry phospholipids suggests water which cannot be removed by our usual methods. DOEP is a cationic lipid transfection reagent used as a component of an in vivo, delivery system for DNA and our hydration results thus may well have relevance to both the lipid-DNA binding and the fusion of these liposomes with cells.

Although we have applied BET theory (Table 1) to the analysis of water adsorption (and desorption) isotherms, further work is necessary to show that this application to phospholipids is valid. BET theory as it was developed [7] is applicable to a given adsorption process provided that three conditions are met: (a) the BET plot for the lower ($p/p_0 < 0.5$) partial vapor pressures of water must be a straight line, (b) the values obtained for the BET constants must be physically reasonable and (c) the temperature behavior must be appropriate. Our results indicate that in many cases a good straight-line BET plot is obtained although not always at the very lowest p/p_0 values. A similar deviation at low values of p/p_0 was also found for other gases on a variety of adsorbents [7] and is ascribed to Eq. 1 breaking down for adsorption on the most active part of the adsorbing surfaces. This, in turn, is thought to arise from the necessity for the heat of adsorption to be at least approximately uniform over the adsorbing surface. E_1, therefore, is to be regarded as the average heat of adsorption on the less active part of the adsorbing surface. The linearity of the BET plots for phospholipids usually begins at p/p_0 values where about one water molecule per phospholipid molecule has been adsorbed.

The BET binding energies of water to the various phospholipids shown in Table 1 are physically reasonable in comparison with the values found for different gases on a variety of adsorbents, as discussed in reference [7]. The E_1 values obtained for strong water binding lipids do not differ greatly amongst themselves, i.e., an approximate range of 10–13 kcal/mol as seen in Table 1; a similar range was found by Hasty for a number of different phosphatidylcholines [10]. These E_1 values are on the order of the hydrogen bond energy for water [13]. We thus speculate that for a number of phospholipids, the first few water molecules adsorbed to the dry lipid film rearrange the surface so as to allow the formation of an 'ice-like', hydrogen bonded water structure at this surface; this speculation would be consistent with our electrical conductivity results, to be discussed in a later publication.

We have applied BET theory to a number of zwitterionic phosphatidylcholines [9] and by criteria (a) and (b), BET was found to be applicable to the five different phosphatidylcholines studied. The temperature characteristics of water adsorption isotherms and the associated BET constants have not yet been fully studied by us, nevertheless, we have used BET theory in this work cognizant of this experimental incompleteness. For the third BET condition, to be valid, one must examine how the BET constants c and v_m change with temperature [7]. The results are used to calculate isotherms at other temperatures; these calculated isotherms can then be compared with appropriate experimental isotherms.

According to the BET approach v_m is the number of water molecules adsorbed per phospholipid molecule in the first 'monolayer' of water [7]. We do not, however, interpret this to be a continuous monolayer of water over the lipid film surface but rather the filling of the initial water binding sites of the phospholipids. This interpretation again is consistent with our electrical measurements where we find a very rapid, exponential increase in the DC electrical conductivity of the lipids as the first few (1–2) water molecules are adsorbed, and then a leveling off of the conductivity increase, with further water adsorption. We ascribe this electrical behavior to the completion of specific hydrogen bonded paths over the lipid bilayer surface. The v_m values for the charged phospholipids are between approx. 1.5 and 2.5 water molecules per phospho-

lipid molecule a range similar to that (1.3 to 3.5) found by Hasty for phosphatidylcholines [10]. Since BPS is a weak water binder, i.e., $E_1 \leq E_L$, we can only estimate v_m and to a first approximation [7], i.e., assuming $E_1 = E_L$, we judge v_m to be at most 0.5 water molecules per phospholipid molecule.

On the basis of our results, we propose that the first few adsorbed water molecules may change the molecular arrangement of the dry lipid film for some phospholipids, forming a 'new' surface. The rearrangement may involve the 'ionization' of charged phospholipids, as the lipid is initially hydrated. This process, although alluded to, was not considered in detail in BET theory [7]. In support of this sequence of events, NMR studies suggest that with increasing hydration of the phospholipid, the N^+ end of the phosphocholine head-group moves farther from the hydrocarbon layer [14]. This movement of the head-group away from the hydrocarbon layer, upon adsorption of these first few water molecules, may well result in the new surface to which we propose BET theory applies. The BET values calculated from the isotherms are then average values and apply to this partially hydrated lipid surface.

In summary, electrically charged phospholipids, as do zwitterionic phospholipids, adsorb water in a manner dependent on their total head-group structure. The presence of a net electrical charge on the head group, may modulate this water adsorption but does not, in itself, predict whether the lipid is a strong or weak water adsorber. The total head-group structure has to be considered, perhaps as it affects the intra/inter molecular hydrogen bonding. BET theory provides a conceptual framework for the analysis of this water adsorption process, at least in some cases. Our results should have applicability to such phenomena as 'stealth liposome' interactions [15,16] and magnetization transfer effects in MRI imaging procedures [17]; both of these processes involve water bound to lipids.

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